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PRINCIPAL INVESTIGATOR: Wing C. Chan, M.D.

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198-6810

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Wing C. Chan, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nebraska Medical Center
Omaha, NE 68198-6810

E-Mail: jchan@unmc.edu

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Epstein Barr virus (EBV) has been implicated as a cofactor in several human malignancies. The possibility that EBV may play a role in the development of breast cancer has been raised in recent years. However, published reports have shown conflicting results. This could be related to the different assays employed and also possible geographical variations in the incidence of this infection. We collected 282 cases of invasive breast carcinomas, as well as 30 normal tissues adjacent to the tumors from 5 different geographical regions (USA, India, Kuwait, China and Saudi Arabia). Conventional polymerase chain reaction (PCR), real-time PCR and EBV-encoded small nonpolyadenylated RNA (EBER-1) in situ hybridization (ISH) were used to study these cases. The data indicate that EBV is present in a small subset of breast carcinomas, however, the high PCR positivity is likely due to the presence of latently infected infiltrating lymphocytes. The incidence and/or the viral load of EBV association with breast cancers were not significantly different among the geographical regions studied.

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INTRODUCTION

Epstein-Barr virus (EBV) is a □-herpes virus and infects more than 90% of the world population (1). EBV is associated with several malignancies such as African Burkitt's lymphoma (2), nasopharyngeal carcinoma (NPC) (3), classical Hodgkin'sdisease (4), post-transplant and AIDS-associated lymphomas (5,6), nasal NK/T cell lymphoma (7), and gastric carcinomas (8). Thus, EBV has been classified as a group-1 carcinogen (9).

Breast cancer is the most frequent malignancy and the leading cause of cancer death among women in Western countries (10). Recent studies have suggested an etiological role for EBV in breast cancer (11-18, 25,28). If substantiated, this would have potential implications for the prevention and treatment of breast cancer. However, these studies remain controversial because other studies have questioned this association (19-23, 26, 27). These conflicting results might have been because of the widely differing methodologies used in these studies and also possible geographical variations in the incidence of this infection.

In an attempt to resolve this dispute, the aims of our study are: 1) To compare if different assays for the detection of EBV provide different endpoints. 2) To compare if there is a geographical variation in the association of EBV with breast cancer samples obtained from the USA, Middle Eastern countries, India and China. 3) To determine if the presence of EBV in these samples is restricted to tumor cells.

For the study of geographical variations, besides 203 cases of primary invasive breast cancers collected from USA, India and Kuwait during the first year period, fifty-six Chinese cases and 23 Saudi Arabia cases were obtained during this second year period. We have replaced the Egyptian cases by Chinese cases for two reasons: one is that we could not obtain cases from Egypt, the other is, there is a lot of evidence showing high incident tumors in south China, such as NPC and nasal NK/T cell lymphoma, having strong association with EBV. To unequivocally determine whether the EBV genome is present in the tumor cells of breast cancers, we combined real-time PCR that has the sensitivity and specificity to ensure accurate EBV genome detection, with laser microdissection system (LMD), enabling the isolation of a pure population of tumor cells for analysis. In parallel, we used in situ hybridization to detect the presence of EBV-encoded small nonpolyadenylated RNA (EBER) and developed immunohistochemistry techniques for detection of EBV gene products in breast cancers.

BODY

All 100 USA tumor samples with 29 normal tissues adjacent to tumors and 79 new breast cancer samples (all paraffin embedded tissues, 56 from China and 23 from Saudi Arabia) were screened for the presence of EBV genome by conventional PCR using primers flanking the EBV nuclear antigen 1 (EBNA-1) region. Positive cases then went to EBER ISH to determine whether EBV expression occurs in the tumor epithelial compartment or in the infiltrating lymphocytes. Real time PCR was also performed in all EBNA-1 PCR positive cases to quantitate the EBV load in breast cancers. Defective heterogeneous (Het) EBV PCR was carried out on USA cases to detect some EBER negative cases harboring the defective EBV genome (20). (For the information of DNA isolation and EBER ISH and primer sequences used in conventional PCR and real time PCR, see Annual report-2002 for this award). Among the 282 breast cancers analyzed for EBNA-1 PCR, the overall positivity is 40%. Twenty- nine normal tissues adjacent to USA breast cancers (#1-29) also showed 24% positive rate. EBNA1 showed a positive ratio from USA of 45%, China 62.5%, Saudi Arabia 56%, India 40% and Kuwait 5%, respectively (Figure 1). Frequencies were not significantly different among the geographical areas studied excepted Kuwait cases that were assayed by Dr. Bhatia, our collaborator, in Saudi Arabia. Whether the low frequency of Kuwait breast cancers for EBV represents a real geographical variance or just due to experimental errors, needs to be reexamined later.

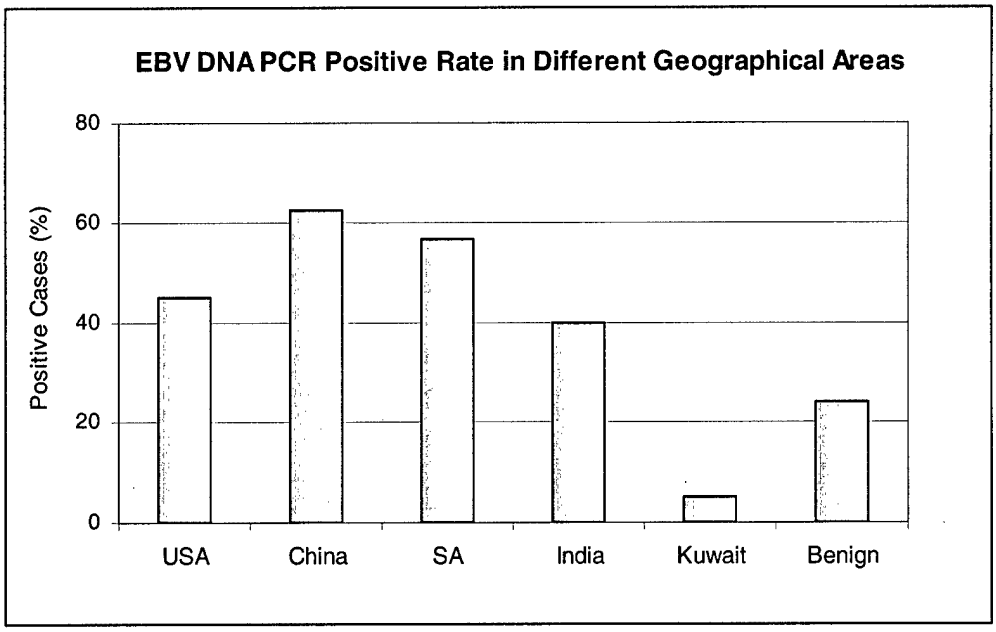

Figure 1: Percentage of EBV genome, as determined by EBNA1 PCR, in breast cancers from different geographical areas. SA- Saudi Arabia.

EBER ISH was carried out on cases identified as EBV positive by whole section PCR. EBER RNA was detected in 12 out of 45 EBNA-1 PCR positive US cases. However, only 4 cases showed positive tumor cell staining. Of which 3 showed focal rare tumor cell staining while 1, US case #39, had up to half of the tumor cells stained strongly with EBER RNAs (figure 2).

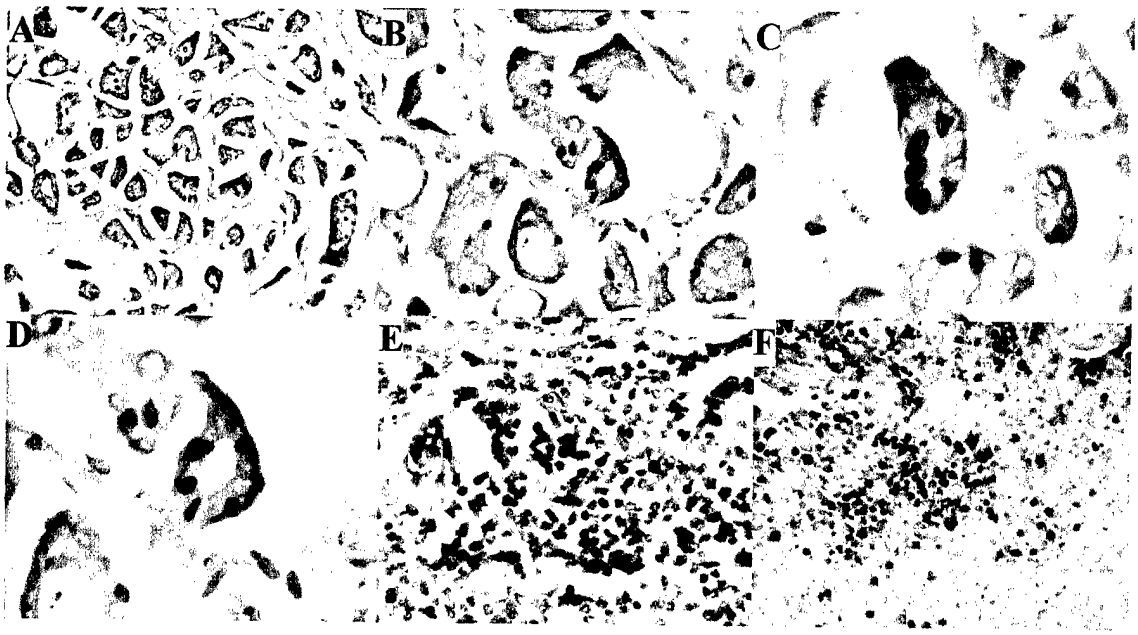

Figure 2. EBER-1 ISH in positive control and breast cancer sections. A, US case #39 shows nuclear staining of tumor cells (10X). B-D, same sections as in A shown in higher power view. E, shows infiltrating lymphocytes in the tumor stroma of the same case, #39, and F, staining on a known EBV positive lymphoma.

These 4 cases all had moderate to heavy lymphocytic infiltration, 3 of the 4 cases also had lymphocytes staining for EBER (Table 1). Among the other 8 EBER ISH positive cases, 3 cases showed only infiltrating lymphocytes staining, 3 cases had staining of only normal surrounding breast glandular cells, 1 case showed many lymphocytes and a few endothelial cells staining and 1 case even showed normal staining of skin epithelial cells (Table 2). None of 29 normal tissues next to tumors were positive with EBER ISH.

Table 1. EBNA1 and Het EBV PCR in breast cancers from different regions.

	EBNA-1 PCR Positive vs total tumor (%)	**Het EBV PCR Positive vs total tumor (%)**
USA	45/100 (45)	11/100 (11)
China	35/56 (62.5)	ND
SA	13/23 (56.5)	ND
India	16/40 (40)	ND
Kuwait	3/63 (5)	ND
Benign	7/29 (24)	2/29 (7)

Four tumor EBER ISH positive cases were also examined for correlation with clinical features (table 3), however, due to the small number of cases, no significant finding can be discerned at this point.

Table 2. Summary of EBER ISH in 12 positive cases.

Case number	Description of EBER ISH staining
#12 A11	Lymphoid cells positive
#17A4	Tumor cells positive
#20A2	Lymphoid cells positive
#38	Few lymphoid cells positive
#39	Tumor cells positive Lymphoid cells positive Focal positive for tumor cells
#41	Few lymphoid cells positive Endothelial cells positive Normal breast glands also positive
#63	Many lymphoid cells positive Endothelial cells positive Few inflammatory cells positive
#68	Rare tumor cells positive Occasional stromal cells positive
#72	Normal skin epithelium shows positive cells
#78	Some surrounding breast glandular cells positive
#82	Some surrounding glandular cells positive
#92	Surrounding glandular epithelium positive Endothelial cells positive

The discrepancy between the PCR and ISH may due to the detection of EBV by PCR on infiltrated lymphocytes. Alternatively, failure of detection of the virus by EBER ISH may reflect low viral load in breast cancer, or there may be deleted EBV in tumor cells that fail to express EBERS (20). In an attempt to answer some of these questions, we developed real-time PCR on BamH1 K fragment to quantitate the EBV DNA copies in breast cancers. Indeed, we detected very low viral load in all EBV PCR positive cases (< 10 copies/1000 cells, data not shown). PCR amplifying the junction of partially deleted and rearranged EBV genome according to Sixby et al, were also done in 100 US cases along with 29 normal tissues next to the tumors. Het PCR detected 11 positive cases out of 100 tumors including 5 cases that were also positive for EBNA-1 PCR. EBNA1 is present in the prototype virus but deleted from het EBV DNA. Two of the 29 normal tissues were also positive for deleted EBV including one which was positive for both the normal and deleted virus (Table 1). However, none of these het EBV positive, prototype virus negative cases were detected positive by EBER ISH.

DNA in situ hybridization can also be used for the localization of the viral genome. However, this method is prone to false-negative results due to the low copy number of the viral genome in latently infected cells. Instead, Laser Microdissection System in combination with real-time PCR will be a better approach to achieve this purpose. These techniques are being developed in our Lab.

Table 3. Correlation of 4 tumor cell positive cases with clinical data.

Case Number	17R	39	41	68
Age Group	(40-50) 50yrs	(>70) 75 yrs	(40-50) 48 yrs	(40-50) 45yrs
Clinical Tumor Size	T2	T1	Tx	T3
Clinical Nodal Status	N1	Nx	Nx	N0
Pathological Tumor Size	4cm	1.8cm	2cm	5.5cm
Pathological Nodal Status	3/14	NA	NA	0/13
Histological Grade	2-3	2	2	3
ER/PR Status	+/+	+/+	+/+	-/-
+Lymphocytic Infiltration Status	Mild	Moderate	Moderate	Heavy

Immunohistochemical methods are available for the detection of viral proteins in paraffin-embedded sections. We obtained antibodies to EBNA-1, latent membrane protein (LMP)-1 and the transactivating immediate-early BZLF1 protein. However, some of these are not consistently expressed in latent EBV infection (). Only EBNA1 is expressed in all known forms of viral latency, yet the antibodies available for the detection of this protein usually produce only weak staining. The most common one used so far, 2B4, was reported to have non-specific reactivity with breast tumor cells in the absence of EBV genome (). Therefore, immunohistochemistry had its limit in terms of studying the presence of EBV in breast cancers. We will optimize the conditions in the immunostaining procedure, meanwhile we will employ the LMD and real-time PCR techniques for the localization of viral genome as described above.

In conclusion, our study showed that using PCR technique alone to detect the presence of EBV genome in breast cancer could obtain 40-60% positive cases. However, PCR cannot distinguish between tumor cells and latently infected lymphocytes. The high rates of positivity may indicate a lack of specificity. In an attempt to define the cellular localization of EBV in breast cancer, the morphology-based assay RNA ISH to detect the expression of EBER was employed. Very few cases turned out to be positive by this assay and in some positive ones, the reactive cells are lymphocytes rather than tumor cells. Therefore more sensitive and accurate methods such as real time PCR in combination with LMD will be a better approach to assess the association of EBV with breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Established EBER in situ hybridization for localization of EBV infected cells.

- Assayed 100 of USA cases, 63 of Kuwait cases, 56 of China cases, 23 of Saudi Arabia cases and 40 of Indian cases of breast carcinoma for EBV by PCR and EBER ISH.

- Developed Real-Time PCR assay for the EBNA1 region and also an assay for the defective EBV.

- Organized a data base for the experimental findings .

- Developed LMD techniques for isolation of tumor cells.

REPORTBLE OUTCOMES:

An abstract titled " Epstein-Barr virus and breast cancer" was presented in the " Era of Hope – Department of Defense Breast Cancer Research Program meeting" on September 24-27, 2002 at Orlando, FL.

The study from this period also generated a manuscript which is currently in preparation.

CONCLUSIONS:

We have examined 282 primary invasive breast cancer cases from various geographical regions including USA (100 cases), Saudi Arabia (23 cases), India (40 cases), China (56cases) and Kuwait (63 cases). All of them were subjected to conventional PCR using the EBNA1 primers. We detected overall positivity of about 40% with this set of primers. The frequencies were not significantly different among the areas studied except Kuwait. The 100 USA cases were also tested for the presence of Het EBV by PCR and this showed a positivity rate of 11%. There were only 5 cases that showed a positivity for both the primers. EBER ISH was carried out on all the PCR positive US cases along with the 29 benign tissues. Twelve cases were positive for EBERs, however, only 4 of these cases showed positive staining in tumor cells, the rest were positive for lymphocytes. Rarely normal glandular cells were found to be positive. This is a interesting finding since we do found the presence of EBV in tumor cells. The origin of these virus may came from infected infiltrating lymphocytes through cell to cell contacts or the neoplastic cells may arise from normal breast glands which harbor EBV. The data overall argue that EBV may play a role in the pathogenesis in rare cases of breast cancer. Our finding was also supported by the report by Xue et al (25). However, EBV was only detected in the tumor cells of very small portion of breast cancers. We will continue our investigation using more sensitive and accurate methods such as real time PCR and Laser Microdissection to determine if there are atypical cases that lack of the expression of EBERs.

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